Docket No. 205645US

TITLE OF THE INVENTION

PROTEINS AND DNA RELATED TO SALT TOLERANCE IN PLANTS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims benefit to U.S. provisional application No 60/194,649, filed on April 04, 2001, and incorporated herein by reference in its entirety.

STATEMENT OF FEDERALLY FUNDED RESEARCH

This invention was supported by the National Institutes of Health by Contract No. R01GM59138 and the U.S. Department of Agriculture by Contract No. 9801270. The government may have certain rights to this invention.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to proteins and nucleic acids related to salt tolerance in plants.

Description of the Background

In *Arabidopsis thaliana*, the Salt Overly Sensitive 7 (SOS2) gene is required for intracellular Na^+ and K^+ homeostasis. Mutations in SOS2 cause Na^+ and K^+ imbalance and render plants more sensitive toward grovleth inhibition by high Na^+ and low K^+ environments. We isolated the SOS2 gene through positional cloning. SOS2 is predicted to encode a serine/threonine type protein kinase with an N-terminal catalytic domain similar to that of the yeast SNF1 kinase. Sequence analyses of *sos2* mutant alleles reveal that both the N-terminal catalytic domain and the C-terminal regulatory domain of SOS2 are functionally essential. The steady-state level of SOS2 transcript is up-regulated by salt stress in the root. Autophosphorylation assays show that SOS2 is an active protein kinase. In the recessive *sos2-5* allele, a conserved glycine residue in the kinase catalytic domain is changed to glutamate. This mutation abolishes SOS2 autophosphorylation, indicating that SOS2 protein

kinase activity is required for salt tolerance.

Control of intracellular ion homeostasis is essential for all cellular organisms. Most cells maintain relatively high K^+ and low Na+ concentrations in the cytosol. In plants. this is achieved through coordinated regulation of transporters for H^+, K^+, and Na^+. At the plasma membrane, a family of P-type H^+-ATPases serves as the primary pump that generates a protonmotive force driving the active transport of other solutes, including K^+ and Na^+ (1). Several plant K^- channels and transporters have been molecularly characterized. The inward rectifying K^+ channel AKT1 is essential for root K^+ uptake in *Arabidopsis thaliana* (2, 3). Expression characteristics indicate that the KAT1 channel is involved in K^+ influx in *Arabidopsis* guard cells (4, 5). Recently, an outward rectifying K^+ channel has been shown to be essential for unloading K^+ into the *Arabidopsis* root xylem (6). The wheat HKT1 gene product functions as a high-affinity K^+ transporter (7). In addition, a family of *KUP* genes exists in *Arabidopsis*. At least one of them, *KUP1*, encodes a protein that can function as a dual-affinity K^+ transporter (8, 9). Na^+ enters plant cells passively, presumably through K^+ transport systems (10), Unlike animals or fungi. plants do not seem to possess Na^+/K^+-ATPases or Na^--ATPases. Na^+ efflux s achieved through the activities of Na^+/H^+ antiporters on the plasma membrane. Much of the Na^+ that enters the cell is compartmentalized into the vacuole through the action of vacuolar Na^+/H^+ antiporters (11,12). The driving force for the vacuolar transporters is the protonmotive force created by vacuolar V-type H^+-ATPases and the H^+-pyrophosphatase (1, 13). Although there has been great progress in the characterization of K^+ and Na^+ transporters in plants, little is currently known about their regulation.

In the trophic chain, plant roots play pivotal roles by taking up mineral nutrients from soil solutions. Plant roots experience constant fluctuations in soil environments. A frequent variant in the soil solution is Na^+ concentration (14). Na^+ is not an essential ion for most plants. In fact, the growth of the majority of plants, glycophytes, is inhibited by the presence of high concentrations of soil Na^+. External Na^+ causes K^+ deficiency by inhibiting K^+ uptake into plant cells (15). Na^+ accumulation within the cell is toxic to many cytosolic enzymes. In contrast, many cellular enzymes are activated by K^+, which is the most abundant cation in the cytoplasm. Certain cytoplasmic enzymes arc especially prone to Na^+ inhibition when K^+ concentration is reduced (16). Therefore, maintaining intracellular K^+ and homeostasis to preserve a high K^+/Na^+ ratio is important for all cells and especially critical for plant cells.

A family of *Arabidopsis sos* (salt overly sensitive) mutants defective in the regulation of intracellular Na^+ and K^+ homeostasis was recently characterized (15, 17, 18). The *sos* mutants are specifically hypersensitive to inhibition by high concentrations of external Na^+ or Li^+ (17, 18). In response to high Na^+ challenge, the *sos2* and *sos3* mutants accumulate more Na^+ and retain less K^+ than wild-type plants (18). The mutants are also unable to grow when the external K^+ concentration is very low (17, 18). These phenotypes suggest that the mutant plants are defective in the regulation of K^+ and Na^+ transport (18). The *SOS3* gene was recently cloned and shown to encode an EF hand-type calcium-binding protein that shares significant sequence similarities with animal neuronal calcium sensors and the yeast calcineurin B subunit (19). In yeast, calcineurin is a central component in the signaling pathway that regulates Na^+ and K^+ homeostasis (20, 21). Loss-of-function mutations in calcineurin B cause increased sensitivity of yeast cells to Na^+ or Li^+ stress.

Because of limited water supplies and the widespread use of irrigation, the soils of many cultivated areas have become increasingly salinized. In particular, modern agricultural practices such as irrigation impart increasing salt concentrations when the available irrigation water evaporates and leaves previously dissolved salts behind. As a result, the development of salt tolerant cultivars of agronomically important crops has become important in many parts of the world. For example, in salty soil found in areas such as Southern California, Arizona, New Mexico and Texas.

Dissolved salts in the soil increase the osmotic pressure of the solution in the soil and tend to decrease the rate at which water from the soil will enter the roots. If the solution in the soil becomes too saturated with dissolved salts, the water may actually be withdrawn from the plant roots. Thus the plants slowly starve though the supply of water and dissolved nutrients may be more than ample. Also, elements such as sodium are known to be toxic to plants when they are taken up by the plants.

Salt tolerant plants can facilitate use of marginal areas for crop production, or allow a wider range of sources of irrigation water. Traditional plant breeding methods have, thus far, not yielded substantial improvements in salt tolerance and growth of crop plants. In addition, such methods require long term selection and testing before new cultivars can be identified.

Accordingly, there is a need to increase salt tolerance in plants, particularly those plants which are advantageously useful as agricultural crops.

SUMMARY OF THE INVENTION

We report here the positional cloning of the *SOS2* locus. *SOS2* is predicted to encode a serine/threonine type protein kinase with an N-terminal catalytic domain highly similar to those of yeast SNF1 and mammalian AMPK kinases. Sequence analyses of several *sos2* mutant alleles point to a functional requirement of both the N-terminal catalytic domain and the C-terminal regulatory domain of *SOS2*. *SOS2* is expressed in both the root and shoot. In the root, *SOS2* mRNA is up-regulated by salt stress. Autophosphorylation assays demonstrate that *SOS2* is an active protein kinase. Furthermore, a mutation that abolishes SOS2 autophosphorylation renders plants hypersensitive to salt stress, indicating that SOS2 protein kinase activity is necessary for salt tolerance. This demonstrates that a protein kinase is essential for intracellular Na^+ and K^+ homeostasis and plant salt tolerance.

Thus, the present invention provides an isolated polynucleotide which encodes a protein comprising the amino acid sequence in SEQ ID NO:2.

In a preferred embodiment the polypeptide has serine/threonine kinase activity.

In another preferred embodiment the polynucleotide comprises SEQ ID NO:1, polynucleotides which are complimentary to SEQ ID NO:1, polynucleotides which are at least 70%, 80% and 90% identical to SEQ ID NO:1; or those sequence which hybridize under stringent conditions to SEQ ID NO:1, the stringent conditions comprise washing in 5X SSC at a temperature from 50 to 68°C.

In another preferred embodiment the polynucleotides of the present invention are in a vector and/or a host cell. Preferably, the polynucleotides are in a plant cell or transgenic plant. Preferably, the plant is Arabidopsis thaliania or selected from the group consisting of wheat, corn, peanut cotton, oat, and soybean plant. In a preferred embodiment, the polynucleotides are operably linked to a promoter, preferably an inducible promoter.

In another preferred embodiment the present invention provides, a process for screening for polynucleotides which encode a protein having serine/threonine kinase activity comprising hybridizing the polynucleotide of the invention to the polynucleotide to be screened; expressing the polynucleotide to produce a protein; and detecting the presence or absence of serine/threonine kinase activity in said protein.

In another preferred embodiment, the present invention provides a method for detecting a nucleic acid with at least 70% homology to nucleotide SEQ ID NO:1, sequences which are complimentary to SEQ ID NO:1 and/or which encode a protein having the amino

acid sequence in SEQ ID NO:2 comprising contacting a nucleic acid sample with a probe or primer comprising at least 15 consecutive nucleotides of the nucleotide sequence of Claim 1, or at least 15 consecutive nucleotides of the complement thereof.

In another preferred embodiment, the present invention provides a method for producing a nucleic acid with at least 70% homology to the polynucleotides of the present invention comprising contacting a nucleic acid sample with a primer comprising at least 15 consecutive nucleotides of the nucleotide sequence of Claim 3, or at least 15 consecutive nucleotides of the complement thereof.

In another preferred embodiment, the present invention provides a method for making SOS2 protein, comprising culturing the host cell carrying the polynucleotides of the invention for a time and under conditions suitable for expression of SOS2, and collecting the SOS2 protein.

In another preferred embodiment, the present invention provides a method of making a transgenic plant comprising introducing the polynucleotides of the invention into the plant.

In another preferred embodiment, the present invention provides method of increasing the salt tolerance of a plant in need thereof, comprising introducing the polynucleotides of the invention into said plant.

In another preferred embodiment, the present invention provides an isolated polypeptide comprising the amino acid sequence in SEQ ID NO:2 or those proteins that are at least 70%, preferably 80%, preferably 90% and preferably 95% identity to SEQ ID NO:2. Preferably, the polypeptides have serine/therenine kinase activity.

BRIEF DESCRIPTION OF THE FIGURES

A more complete appreciation of the invention and many of the attendant advantages thereof will be readily obtained as the same becomes better understood by reference to the following detailed description when considered in connection with the accompanying drawings, wherein:

Fig. 1: Positional cloning of the SOS2 gene. (A) Physical mapping of SOS 2. Genetic mapping delimited SOS2 to a region in the BAC clone K21B8. The SOS2 gene was identified by sequencing candidate genes in this region from sos2 mutant and wild-type plants. (B) Structure of SOS2 and position of sos2 mutations. Positions are relative to the initiation codon. Filled boxes indicate the ORF, and the lines between boxes indicate introns.

Fig. 2: SOS2 encodes a putative serine/threonine protein kinase. (A) Diagrammatic representation of SOS2 structure. (B) SOS2 cDNA sequence and the conceptual translation product of its longest ORF (GenBank accession number AF 237670). Underlined is a stop codon (TAA) at -6 to -4 that precedes the ATG in-frame. Numbers I-XI indicate kinase subdomains as defined by Hanks et al. (25), with invariant and nearly invariant amino acid residues highlighted in black and gray, respectively.

Fig. 3: Amino acid alignments. (A) Alignment of putative kinase catalytic domain of SOS2 with *Saccharomyces cerevisiae* SNF1 (23) and human AMPK kinases (24). Amino acid residues identical in at least two proteins are highlighted in black and conservative substitutions in gray. Mutations that abolish SOS2 autophosphorylation (see Fig. 4) are indicated; first * is K40N,m and second is * G197E, which corresponds to the *sos2-5 allele.* (B) Alignment of the C-terminal portion of SOS2 with the regulatory domains of *Schizosaccharomyces pombe* (yCHK1) and human CHK1 (hCHK1) kinases (27).

Fig. 4: Autophosphorylation of SOS2 kinase. GST, GST-SOS2, GST-SOS2 plus GST-SOS3, and mutated kinases GST-SOS2 (K40N) and GST-SOS2(G187E) were expressed in *E. coli*, purified from bacterial lysates by means of glutathione Sepharose chromatography, incubated with [γ-^{32}p] ATP in kinase buffer, electrophoresed on SDS/polyacrylamide gel, and Coomassie stained (Left), and exposed to x-ray film (Right).

Fig. 5: Regulation of SOS2 expression by salt stress. Plants were treated with 200 mM NaCl (A) or with nutrient solutation as a control (B) for the indicated time periods. Total RNA were extracted from roots and shoots, and subjected to Northern blot analysis with ^{32}P-labeled SOS2 cDNA as probe. Thirty-five micrograms of total RNA was loaded in each lane. Ethidium bromide-stained rRNA bands were used as controls for equal loading.

DETAILED DESCRIPTION OF THE INVENTION

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art of molecular biology. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described herein. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods,

and examples are illustrative only and are not intended to be limiting.

Reference is made to standard textbooks of molecular biology that contain definitions and methods and means for carrying out basic techniques, encompassed by the present invention. See, for example, Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York (1982) and Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York (1989); Methods in Plant Molecular Biology, Maliga et al, Eds., Cold Spring Harbor Laboratory Press, New York (1995); Arabidopsis, Meyerowitz et al, Eds., Cold Spring Harbor Laboratory Press, New York (1994) and the various references cited therein.

The term "plant" includes whole plants, plant organs (e.g., leaves, stems, roots, etc.), seeds and plant cells and progeny of same. The class of plants which can be used in the methods of the invention is generally as broad as the class of higher plants amenable to transformation techniques, including both monocotyledonous and dicotyledonous plants. Preferred plants include rice, corn, wheat, cotton, peanut, and soybean.

Thus, in one embodiment of the present invention, the salt tolerance of a plant can be enhanced or increased by increasing the amount of protein available in the plant, preferably by the enhancement of the SOS2 gene in the plant.

Thus, one embodiment of the present invention are plant cells carrying the polynucleotides of the present invention, and preferably transgenic plants carrying the isolated polynucleotides of the present invention.

As used herein, the term "enhancement" means increasing the intracellular activity of one or more enzymes in a plant cell and/or plant which are encoded by the corresponding DNA. Enhancement can be achieved with the aid of various manipulations of the bacterial cell. In order to achieve enhancement, particularly over-expression, the number of copies of the corresponding gene can be increased, a strong promoter can be used, or the promoter- and regulation region or the ribosome binding site which is situated upstream of the structural gene can be mutated. Expression cassettes which are incorporated upstream of the structural gene act in the same manner. In addition, it is possible to increase expression by employing inducible promoters. A gene can also be used which encodes a corresponding enzyme with a high activity. Expression can also be improved by measures for extending the life of the

mRNA. Furthermore, enzyme activity as a whole is increased by preventing the degradation of the enzyme. Moreover, these measures can optionally be combined in any desired manner. These and other methods for altering gene activity in a plant are known as described, for example, in Methods in Plant Molecular Biology, Maliga et al, Eds., Cold Spring Harbor Laboratory Press, New York (1995).

A gene can also be used which encodes a corresponding or variant enzyme with a high activity. Preferably the corresponding enzyme has a greater activity than the native form of the enzyme, more preferably at least in the range of 5, 10, 25% or 50% more activity, most preferably more than twice the activity of the native enzyme.

In the context of the present Application, a polynucleotide sequence is "homologous" with the sequence according to the invention if at least 70%, preferably at least 80%, most preferably at least 90% of its base composition and base sequence corresponds to the sequence according to the invention. According to the invention, a "homologous protein" is to be understood to comprise proteins which contain an amino acid sequence at least 70 % of which, preferably at least 80 % of which, most preferably at least 90 % of which, corresponds to the amino acid sequence which is encoded by the SOS2 gene (SEQ ID No.1), wherein corresponds is to be understood to mean that the corresponding amino acids are either identical or are mutually homologous amino acids. The expression "homologous amino acids"denotes those which have corresponding properties, particularly with regard to their charge, hydrophobic character, steric properties, etc.

Homology, sequence similarity or sequence identity of nucleotide or amino acid sequences may be determined conventionally by using known software or computer programs such as the *BestFit* or *Gap* pairwise comparison programs (GCG Wisconsin Package, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin 53711). *BestFit* uses the local homology algorithm of Smith and Waterman, Advances in Applied Mathematics 2: 482-489 (1981), to find the best segment of identity or similarity between two sequences. *Gap* performs global alignments: all of one sequence with all of another similar sequence using the method of Needleman and Wunsch, J. Mol. Biol. 48:443-453 (1970). When using a sequence alignment program such as *BestFit*, to determine the degree of sequence homology, similarity or identity, the default setting may be used, or an appropriate scoring matrix may be selected to optimize identity, similarity or homology

scores. Similarly, when using a program such as *BestFit* to determine sequence identity, similarity or homology between two different amino acid sequences, the default settings may be used, or an appropriate scoring matrix, such as *blosum45* or *blosum80*, may be selected to optimize identity, similarity or homology scores.

The present invention also relates to polynucleotides which contain the complete gene with the polynucleotide sequence corresponding to SEQ ID No. 1 or fragments thereof, and which can be obtained by screening by means of the hybridization of a corresponding gene bank with a probe which contains the sequence of said polynucleotide corresponding to SEQ ID No. 1 or a fragment thereof, and isolation of said DNA sequence.

Polynucleotide sequences according to the invention are suitable as hybridization probes for RNA, cDNA and DNA, in order to isolate those cDNAs or genes which exhibit a high degree of similarity to the sequence of the SOS2 gene.

Polynucleotide sequences according to the invention are also suitable as primers for polymerase chain reaction (PCR) for the production of DNA which encodes an enzyme having activity of a serine/threonine kinase.

Oligonucleotides such as these, which serve as probes or primers, can contain more than 30, preferably up to 30, more preferably up to 20, most preferably at least 15 successive nucleotides. Oligonucleotides with a length of at least 40 or 50 nucleotides are also suitable.

The term "isolated" means separated from its natural environment.

The term "polynucleotide" refers in general to polyribonucleotides and polydeoxyribonucleotides, and can denote an unmodified RNA or DNA or a modified RNA or DNA.

The term "polypeptides" is to be understood to mean peptides or proteins which contain two or more amino acids which are bound via peptide bonds.

The polypeptides according to invention include polypeptides corresponding to SEQ ID No. 2, particularly those with the biological activity of a serine/threonine kinase, and also includes those, at least 70 % of which, preferably at least 80% of which, are homologous with the polypeptide corresponding to SEQ ID No. 2, and most preferably those which

exhibit a homology of least 90 % to 95 % with the polypeptide corresponding to SEQ ID No. 2 and which have the cited activity.

The invention also relates to coding DNA sequences which result from SEQ ID No. 1 by degeneration of the genetic code. In the same manner, the invention further relates to DNA sequences which hybridize with SEQ ID No. 1 or with parts of SEQ ID No. 1. Moreover, one skilled in the art is also aware of conservative amino acid replacements such as the replacement of glycine by alanine or of aspartic acid by glutamic acid in proteins as "sense mutations" which do not result in any fundamental change in the activity of the protein, i.e. which are functionally neutral. It is also known that changes at the N- and/or C-terminus of a protein do not substantially impair the function thereof, and may even stabilise said function.

In the same manner, the present invention also relates to DNA sequences which hybridize with SEQ ID No. 1 or with parts of SEQ ID No. 1. Finally, the present invention relates to DNA sequences which are produced by polymerase chain reaction (PCR) using oligonucleotide primers which result from SEQ ID No. 1. Oligonucleotides of this type typically have a length of at least 15 nucleotides.

The terms "stringent conditions" or "stringent hybridization conditions" includes reference to conditions under which a polynucleotide will hybridize to its target sequence, to a detectably greater degree than other sequences (e.g., at least 2-fold over background). Stringent conditions are sequence-dependent and will be different in different circumstances. By controlling the stringency of the hybridization and/or washing conditions, target sequences can be identified which are 100% complementary to the probe (homologous probing). Alternatively, stringency conditions can be adjusted to allow some mismatching in sequences so that lower degrees of similarity are detected (heterologous probing).

Typically, stringent conditions will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C. for short probes (e.g., 10 to 50 nucleotides) and at least about 60°C. for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary low stringency conditions include hybridization with a buffer

solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at 37°C., and a wash in 1X to 2X SSC (20X SSC=3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55°C. Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1 M NaCl, 1% SDS at 37°C., and a wash in 0.5X to 1X SSC at 55 to 60°C. Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37°C., and a wash in 0.1X SSC at 60 to 65°C.

Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. For DNA--DNA hybrids, the Tm can be approximated from the equation of Meinkoth and Wahl, Anal. Biochem., 138:267-284 (1984): Tm =81.5oC.+16.6 (log M)+0.41 (%GC)-0.61 (% form)-500/L; where M is the molarity of monovalent cations, %GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. The Tm is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched probe. Tm is reduced by about 1°C. for each 1% of mismatching; thus, Tm, hybridization and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with approximately 90% identity are sought, the Tm can be decreased 10 °C. Generally, stringent conditions are selected to be about 5 °C lower than the thermal melting point (Tm) for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or 4 °C. lower than the thermal melting point (Tm); moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or 10 °C. lower than the thermal melting point (Tm); low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20 °C. lower than the thermal melting point (Tm). Using the equation, hybridization and wash compositions, and desired Tm, those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a Tm of less than 45 °C. (aqueous solution) or 32 °C. (formamide solution) it is preferred to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Current Protocols in Molecular Biology, Chapter 2, Ausubel, et al., Eds., Greene Publishing and Wiley-Interscience, New York (2000).
Thus, with the foregoing information, the skilled artisan can identify and isolated

polynucleotides which are substantially similar to the present polynucleotides. In so isolating such a polynucleotide, the polynucleotide can be used as the present polynucleotide in, for example, increasing the salt tolerance of a plant.

One embodiment of the present invention is methods of screening for polynucleotides which have substantial homology to the polynucleotides of the present invention, preferably those polynucleotides encoding a protein having serine/threonine kinase activity.

The polynucleotide sequences of the present invention can be carried on one or more suitable plasmid vectors, as known in the art for plants or the like.

In one embodiment, it may be advantageous for propagating the polynucleotide to carry it in a bacterial or fungal strain with the appropriate vector suitable for the cell type. Common methods of propagating polynucleotides and producing proteins in these cell types are known in the art and are described, for example, in Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York (1982) and Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York (1989).

Materials and Methods

Genetic and Physical Mapping. Genetic mapping with restriction fragment length polymorphism and PCR-based markers was as described (19). Construction of yeast artificial chromosome (YAC) and bacterial artificial chromosome (BAC) clone contigs (1) was partly based on information available at http://www.nucleus.cshl.org/protarab/chrom5.map and http://www.kazusa.or.jp/arabi/chr5/map/12-14Mb. This information is incorporated herein by reference.

Nucleic Acid Analysis. For sequence determination, DNA was amplified from wild-type plants and *sos2* mutants by PCR. Nine *sos2* mutants alleles are known (18). All of the alleles were analyzed except *sos24* and *sos2-9* because viable seeds were not available. To avoid errors resulting from PCR, the products of five independent PCRs were pooled and sequenced. Reverse transcription-PCR was carried out on mRNA isolated from 2-week-old

Arabidopsis seedlings. Salt stress treatment, RNA extraction, and Northern blot analysis were carried out as described by Ishitani et al. (22).

Protein Expression. To produce bacterially expressed recombinant proteins, the coding region of *SOS2*, SOS2(K40N), and SOS2(G197E) cDNAs were amplified by PCR with primers harboring restriction sites, cloned in frame into *Bam*HI-*Eco*RI of pGEX-2TK (Amersham Pharmacia), and transformed into *Escherichia coli* BL21 DE3 cells (Amersham Pharmacia). Mutations K40N and G197E in the SOS2 protein were created by site-directed mutagenesis. For glutathione S-transferase (GST)-SOS2(K40N), primer pairs 5'-GCGGATCCATGACAAAGAAAATGAGAAGAGTGGGC and 5'-ATTGTACTCTTAGCCATAATGTTGATGGCT were used for the first PCR, and 5'-GCGAATTCTTAAGTTGGGATCAAAACGTGATTGTTCTG and 5'-GTGATAATGTAGCCATCAACATTATGGCTA were used for the second PCR. For the mutant protein GST-SOS2 (G197E), primer pairs 5'-GCGGATCCATGACAAAGAAAATGAGAAGAGTGGGC and 5'-ATATAACGAAAAGAATAACCTCGCAAGACC were used for the first reaction and 5'-GCTGATATTTGGTCTTGCGAGGTTATTCTT and 5'-GCGAATTCTTAAGTTGGGATCAAAACGTGATTGTTCTG were used for the second reaction. The final amplification was done with 5'-GCGGATCCATGACAAAGAAAATGAGAAGAGTGGGC and 5'-GCGAATTCTTAAGTTGGGATCAAAACGTGATTGTTCTG on both templates. The final constructs were confirmed by sequencing *E. coli* cultures were induced with 0.5 mM isopropyl β-D-thiogalactoside, and recombinant proteins were affinity-purified from bacterial lysates with glutathione-Sepharose beads (Amersham Pharmacia).

Kinase Assay. GST-fusion proteins were incubated in kinase buffer [20 mM Tris-HCl (pH 8.0)/5 mM $MgCl_2$/1 mM $CaCl_2$/1 mM DTT]. The kinase reaction was started by adding [γ-^{32}P]ATP and was trasnferred to 30°C for 30 min. The reaction was stopped by adding 4X SDS-sample buffer and analyzed by SDS/PAGE and autoradiography.

Results

Positional Cloning of SOS2. The SOS2 gene was mapped by crossing the *sos2-2/sos2-2* mutant, which is in the Columbia ecotype, to the *SOS2/SOS2* Landsberg ecotype. On the basis of analysis of 1230 recombinant chromosomes, the SOS2 locus was previously mapped to chromosome V, between molecular markers *nga76* and *PHYC* (18). Further genetic mapping using the recombination crossover points narrowed *SOS2* to a region between the restriction fragment length polymorphism markers mi291b and GSL (Fig. 1A). A YAC contig covering this region was assembled. The left end of YAC CIC4B3 (LE4B3) was found to be tightly linked to SOS2 because no recombination occurred (Fig. 1A). A contig of BAC clones centered around LE4B3 was assembled. Simple sequence length polymorphism markers 26D22-1 and MOK9-3 and single nucleotide polymorphism marker LEMOK9 were developed based on sequence information of the respective BAC clones. Genetic mapping using these markers delimited the SOS2 locus to a 60-kb region of K21B8. Sequence analysis revealed that a candidate gene within this region carries a 2-bp deletion in the sos2-2 allele, which was generated by fast neutron irradiation (Fig. 1B). Further sequence analyses revealed that other sos2 alleles all carry mutations in this gene (Fig. 1B). Each mutation causes a change in amino acid sequence in the predicted ORF. We therefore conclude that this candidate is the SOS2 gene.

SOS2 Encodes a Protein Kinase. The transcribed sequence of the SOS2 gene was determined by sequencing cDNAs obtained by reverse transcription-PCR. Comparison with the genomic sequence showed that the SOS2 gene contains 13 exons and 12 introns (Fig. 1B). SOS2 is predicted to encode a protein of 446 amino acids with an estimated molecular mass of 51 kDa (Fig. 2). Database searches revealed that the deduced amino acid sequence of SOS2 has similarity with various serine/threonine protein kinases. The putative kinase catalytic domain of SOS2 resides in the N-terminal portion of the protein (Fig. 2A) and contains the 11 subdomains common to protein kinases (25). The putative catalytic domain sequence is most similar to the yeast SNF1 and mammalian AMPK kinases (23, 24) (Fig. 3A). The sos2-5, sos2-6, sos2-7, and sos2-8 mutations are predicted to disrupt the kinase catalytic domain. In the sos2-5 allele, Gly-197, which corresponds to one of the invariant amino acid residues of subdomain IX of protein kinases (25, 26), is changed to a negatively charged glutamic acid residue. sos2-6, sos2-7, and sos2-8 are identical mutations that disrupt

the donor site of an intron splice junction (Fig. 1B), resulting in mal-splicing, premature termination, and a truncated polypeptide of 130 amino acid residues.

The C-terminal putative regulatory domain of SOS2 is relatively unique. Part of this domain of SOS2 shows low sequence homology with the regulatory domains of DNA replication checkpoint kinase CHK1 from yeast and humans (27-29) (Fig. 3B). Analysis of the other sos2 mutant alleles revealed that the mutations disrupt only the putative regulatory domain, leaving the catalytic domain intact. This suggests an essential function of the putative regulatory domain in plant Na^+ tolerance. In the sos2-1 mutant allele, a single base pair substitution at the acceptor site of an intron splicing junction results in the addition of 29 amino acid residues between Glu-390 and Ile-391, presumably disrupting the function of the putative regulatory domain. The sos2-2 mutation that was created by fast neutron bombardment (18) has a 2-bp deletion that causes frameshift and premature termination, resulting in a truncated polypeptide of 287 amino acids. In the sos2-3 mutant allele, a single nucleotide substitution creates a stop codon that truncates the protein at Pro-262.

Protein Kinase Activity is Required for SOS2 Function. To determine whether SOS2 encodes a functional protein kinase, the SOS2 ORF was cloned into pGEX-2TK and expressed in bacteria as a C-terminal fusion protein to the bacterial GST. GST-SOS2 was purified from bacterial lysate by affinity chromatography with glutathione-Sepharose beads and shown to have the expected molecular mass of 78 kDa (Fig. 4). Incubation of the recombinant protein with [γ^{32}P]ATP in an *in vitro* kinase assay produced a strong phosphorylation signal that is likely the result of SOS2 autophosphorylation (Fig. 4, lanes 2 and 3). Lys-40 of SOS2 corresponds to a highly conserved residue in subdomain II (Fig. 2) that is required for activity in most protein kinases (25). To verify that the phosphorylation signal was because of SOS2 autophosphorylation, Lys-40 was changed to Asn by means of site-directed mutagenesis and the resulting mutant GST-SOS2(K40N) subjected to autophosphorylation assays. The Lys-40 to Asn mutation abolished the autophosphorylation of SOS2 (Fig. 4, lane 4).

In the sos2-5 mutant allele, the highly conserved Gly-197 is changed to Glu. We expressed the sos2-5 allele in bacteria, and the resulting mutant protein GST-SOS2(G197E) was examined for kinase activity. Like the Lys-40Asn mutation, the sos2-5 mutation also abolished the autophosphorylation activity of SOS2 (Fig. 4, lane 5). Because sos2-5 is a

recessive mutation (18), the results show that kinase activity is required for SOS2 function in plant salt tolerance.

SOS2 kinase apparently has a very specific substrate requirement because none of the commonly used protein kinase substrates, such as histone H1, myelin basic protein, and casein, was phosphorylated by SOS2 (data not shown). SOS3 was not phosphorylated by SOS2, nor did it appear to affect SOS2 autophosphorylation *in vitro* (Fig. 4, lane 3). We have recently found several synthetic serine- or threonine-containing peptides that can be readily phosphorylated by SOS2 (30). In addition, phosphorylation of the peptides by SOS2 depended on the presence of both SOS3 and Ca^{2+} (30).

SOS2 Expression in the Root Is Up-Regulated by Salt Stress. To analyze SOS2 expression under salt stress, 10-day-old *Arabidopsis* seedlings in agar plates were pulled out of agar medium and placed on filter papers soaked with 200 mM NaCl in Murashige and Skoog nutrient solution for 3, 6, or 12 h. Control plants were treated in the same manner, except no NaCl was added to the nutrient solution. After the treatment, roots and shoots were separated at the base of hypocotyls. Total RNA was extracted from control and NaCl-treated tissues. Northern blot analysis using SOS2 cDNA as a probe detected a single transcript of approximately 1.5 kb, the expected size of SOS2 mRNA (Fig. 5). In the root, a very low level of SOS2 mRNA was present in the sample before salt treatment (0 h). After 6 or 12 h of NaCl treatment, increased levels of SOS2 mRNA were detected (Fig. 5A). In the shoot, slight up-regulation of SOS2 transcript was found after 3 or 6 h of NaCl treatment. However, 12 h of NaCl treatment appeared to decrease SOS2 expression in the shoot (Fig. 5A). In the control treatments, only very low levels of SOS2 transcript were detected, and no up-regulation could be seen throughout the time course (Fig. 5B). Overall, the steady-state level of SOS2 transcript was very low, and it took approximately a week of x-ray exposure to obtain the signals shown, whereas a few hours were enough for most other stress-induced genes.

Discussion

SOS2 is a major salt tolerance locus in *A. thaliana* (18). Mutations in the SOS2 gene drastically reduce plant tolerance to high Na^+ stress and to low K^+ stress. Based on mutant

characterization, we have postulated previously that SOS2 might encode a regulatory protein that controls the expression and/or activities of certain K^++ and Na+ transporters (18). In the present study, we have isolated the SOS2 gene through positional cloning. Indeed, SOS2 encodes a regulatory protein, a protein kinase. Protein phosphorylation is a frequent theme of cellular signal transduction, and its involvement in plant ion homeostasis and salt tolerance has been hypothesized (31). Our results provide direct evidence that protein phosphorylation is involved in Na^+ and K^+ homeostasis and plant salt tolerance. Future identification of protein substrate(s) that are phosphorylated by the SOS2 kinase will help understand how plant salt tolerance is regulated by protein phosphorylation. Candidate physiological substrates of SOS2 might include K^+ and Na^+ transporters and/or transcription factor(s) that control their expression.

The similar phenotypes of sos2 and sos3 mutants suggested that SOS2 and SOS3 may function in the same regulatory pathway (18). We tested and found that SOS3 is not phosphorylated by SOS2, nor did it affect SOS2 autophosphorylation (Fig. 4). Nevertheless, we have discovered that SOS2 physically interacts with SOS3, and SOS2 phosphorylation of peptide substrates is activated by SOS3 in a calcium-dependent manner (30).

SOS2 transcript is present in both roots and shoots. This is consistent with the observation that both the root and the shoot of sos2 mutant plants are hypersensitive to NaCl stress (18). SOS2 expression in the root appears to be up-regulated by NaCl stress. The significance of this up-regulation is unclear. There is certainly a very low level of expression in the root even without stress treatment, which could be detected by very long exposures in the Northern blot analysis or by reverse transcription-PCR (data not shown). The expression of SOS2 in the absence of stress is consistent with its role in primary signal transduction leading to salt adaptation. The slight up-regulation of SOS2 transcript may be important to maintain a sufficient level of SOS2 protein during salt stress. Like SOS2, SOS3 expression level is also very low (J.-K.Z., unpublished results). This probably reflects that SOS3 and SOS2 play regulatory roles that do not necessarily require abundant expression.

SOS2 encodes a protein kinase with a catalytic domain at the N terminus and a regulatory domain at the C terminus. The kinase catalytic domain is essential for SOS2 function. The sos2-5 mutation causes a single amino acid substitution within the catalytic domain that abolishes kinase autophosphorylation, resulting in the loss of SOS2 function and

therefore increased sensitivity to salt stress. The regulatory domain also appears to be essential for SOS2 function because mutations that truncate (sos2-2 and sos2-3) or disrupt this domain (sos2-1) render plants hypersensitive to high Na^+ and low K^+ stresses. The catalytic domain of SOS2 is highly similar to the catalytic domains of SNF1/AMPK kinases (Fig. 3A). SNF1/AMPK kinases function to protect cells against nutritional or environmental stresses that deplete cellular ATP by regulating both metabolism and expression (23, 24). Although the catalytic domain of SO is very similar to those of year SNF1 and mammalian AMPK kinases, SOS2 clearly is not a plant homolog of SNF1/AMPK. This is because true plant SNF1/AMPK kinases, such as SnRK1 share substantial sequence similarity with yeast SNF1 and mammalian AMPK at the C-terminal regulatory domain in addition to very high similarity at the N-terminal catalytic domain (32). Part of the regulatory domain of SOS2 is similar to the DNA repair and replication checkpoint kinase CHK1 (Fig. 3B) which is required for cell cycle arrest in response to DNA damage (27-29). The sequence similarity with CHK1 kinase is interesting because sos2 mutants show cell cycle defect at the root meristem in the presence of Na^+ stress (J.-K.Z., unpublished data).

Although several protein kinases were previously reported to play roles in plant stress responses, none of them functions in ion homeostasis (33-36). The AtDBF2 protein kinase was identified by its ability to increase not only salt tolerance but also osmotic heat and cold stress tolerance when overexpressed in *Saccharomyces cerevisiae* or in cultured tobacco cells (33). The mitogen activated protein kinase MKK4 from alfalfa was shown to be activated by cold and drought but not by salt stress (34). Extopic expression of a calcium-dependent protein kinase in maize protoplasts activates the expression of cold- and abscisic responsive genes (36). The transcript levels of several protein kinases were shown to be up-regulated by various stresses including touch, cold, and osmotic stress (35); however, functions remain unknown. In contrast to these previously reported protein kinases that are involved in either general responses or in osmotic and cold stress responses, the SOS2 kinase has specific roles in plant adaptation to high Na^+ and K^+ stresses (18).

Obviously, numerous modifications and variations of the present invention are possible in light of the above teachings. It is therefore to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described herein.

REFERENCES

1. Sze, H., Li, X. & Palmgren, M.G. (1999) *Plant Cell* 11, 677-689.
2. Sentenac, H., BVonnenud, N., Minet, M., Lacroute, F., Salmonm, J.M., Gay-nard, F. & Grignon, C. (1992) *Science* 256, 663-665.
3. Hirsch, R.E., Lewis, B.D., Spadling, E.P. & Sussman, M.R. (1998) *Science* 280, 918-921.
4. Anderson, J.A., Huprikar, S.S., Kochian, L.V., Lucas, W.J. & Gaber, R.F. (1992) *Proc. Natl. Acad. Sci. USA* 89, 3736-3740.
5. Nakamura, R.L., Mckendree, W.L., Jr., Hirsch, R.E., Sedbrook, J.C., Gaber, R. & Sussman, M.R. (1995) *Plant Physiol.* 109, 371-374.
6. Gaymard, F., Pilot, G. Lacombe, B., Bouchez, D., Bruneau, D., Boucherez, J., Michaux-Ferriere, N., Thibaud, J.B. & Sentenac, H. (1998) *Cell* 94, 647-655.
7. Rubio, F., Gassmann, W. & Schroeder, J.I. (1995) *Science* 270, 1660-1663.
8. Kim, E.J., Kwak, J.M., Uozumi, N. & Schoreder, J.I. (1998) *Plant Cell* 10, 61-62.
9. Fu, H.-H. & Lunn, S. (1998) *Plant Cell* 10, 63-73.
10. Schroeder, J.I., Ward, J.M. & Gassmann, W. (1994) *Annu. Rev. Biophys. Biomol. Struct.* 23, 441-471.
11. Gaxiola, R.A., Rao, R., Sherman, A., Grisafi, P., Alper. Sl.L. & Fink, G.R. (1999) *Proc. Natl. Acad. Sci. USA* 96, 1480-1485.
12. Apse, M.P. Aharon, G.S., Soedden, W.A. & Blumwald, E. (1999) *Science* 285, 1256-1258.
13. Rea, P.A. & Poole, R.J. (1993) *Annu. Rev. Plant Physiol. Plant Biol.* 44, 157-180.
14. Epstein, E., Norlyn, J.D., Rush, D.W., Kingsbury, R.W., Kelley, D.B., Cunningham, G.A. & Wronn, A.F. (1980) *Science* 210, 399-404.
15. Wu, S.U., Ding, L. & Zhu, J.K. (1996) *Plant Cell* 8, 617-627.
16. Murguia, J.R., Belles, J.M. & Serrano, R. (1995) *Science* 267, 232-234.
17. Liu, J. & Zhu, J.K. (1997) *Proc. Natl. Acad. Sci. USA* 94, 14960-14964.
18. Zhu, J.-K., Liu, J. & Xiong, L. (1998) *Plant Cell* 10, 1181-1192.
19. Liu, J. & Zhu, J.-K. (1998) *Science* 280, 1943-1945.
20. Nakamura, T. Y., Liu, Y., Hirara, D., Namba, H., Harada, S., Hirokawa, Miyakawa, T. (1993) *EMBO J.* 12, 4063-4071.
21. Mendoza, I., Rubio, F., Rodriguez-Navarro, A., & Pardo, J.M. (1994) *J Biol Chem.* 269, 8792-8796.

22. Ishitani, M., Xiong, L., Stevenson, B. & Zhu, J.-K (1997) *Plant Cell* 1935-1949.

23. Celenza, J.L. & Carlson, M. (1986) *Science* 233, 1175-1180.

24. Mitchelhill, K.L. Stapleton, D., Gao, G., House, C. Mitchell, B., Ks. Witters, L.A. & Kemp, B.E., (1994) *J. Biol. Chem.* 269, 2361-2364.

25. Hanks, S.K., Quinn, A.M. & Hunter, T. (1988) *Science* 241, 42-52.

26. Hanks, S.K. & Hunter, T., (1995) in *The Protein Kinase Facts Book*, eds. D. & Hanks, S. (Academic, London), Vol. 1, pp. 7-47.

27. Walworth, N., Davey, S. & Beach, D. (1993) *Nature* (*London*) 363, 368-371.

28. Walworth, N.C. & Bernards, R. (1996) *Science* 271, 353-356.

29. Boddy, M.N., Furnari, B., Moadesert, O. & Russell, P. (1998) *Science* 280: 909-912.

30. Halfter, U., Ishitani, M. & Zhu, J.-K. (2000) *Proc. Natl. Acad. Sci. USA*. 97(7):3735-40.

31. Niu, X., Bressan, R.A., Hasegawa, P.M. & Pardo, J.M. (1995) *Plant Physiol* 109, 735-742.

32. Halford, N.G. & Hardle, D.G. (1998) *Plant Mol. Biol.* 27, 735-748.

33. Lee, J.H., Van Moningu, M. & Verbruggen, N. (1999) *Proc. Natl. Acad Sci USA* 96, 5873-5877.

34. Jonnk, C., Kiegerl, S., Lighterink, W., Barker, P.J., Huskisson, N.S.H. (1996) *Proc. Natl. Acad. Sci. USA* 93, 11274-11279.

35. Mizoguchi, T., Irie, K., Hirayama, T., Hayashida, N., Yamaguchi-Shino, Matsumoto, K. & Shinozaki, K. (1996) *Proc. Natl. Acad. Sci. USA* 93:765-769.

36. Sheen, J. (1996) *Science* 274, 1900-1902.